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Original article

Phylogeography and historical demography of the neotropical stingless bee *Melipona quadrifasciata* (Hymenoptera, Apidae): incongruence between morphology and mitochondrial DNA*

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Abstract – The stingless bees are among the most abundant and ecologically important social invertebrates in tropical communities. The Neotropical stingless bee *Melipona quadrifasciata* has two subspecies: *M. quadrifasciata quadrifasciata* and *M. quadrifasciata anthidioides*. The main difference between subspecies are the yellow metassomal stripes, which are continuous in *M. q. quadrifasciata* and discontinuous in *M. q. anthidioides*. Recently, two populations were described with continuous stripes and inhabiting clearly disjunct areas in relation to *M. q. quadrifasciata*. We sequenced 852 bp of the mtDNA COI gene from 145 colonies from 56 localities, and for the first time performed a detailed phylogeographic study of a neotropical stingless bee. Phylogenetic analyses revealed the existence of two clades exhibiting a south to north distribution: southern populations comprise the subspecies *M. q. quadrifasciata*, and northern populations are composed of *M. q. anthidioides* and two disjunct populations with continuous stripes. The divergence time of these two phylogroups was estimated between 0.233 and 0.840 million years ago in the Pleistocene, a period of climatic changes and geomorphological alterations in the Neotropical region. No evidence of genetic structure in relation to the tergal stripes was found, indicating that the morphological trait regarding the pattern of stripes on tergites is not an accurate diagnostic for the subspecies of *M. quadrifasciata*.

biogeography / coalescence / tergal stripes / *Melipona quadrifasciata* / subspecies

1. INTRODUCTION

The stingless bee *Melipona quadrifasciata*, popularly known as "mandaçaia", is distributed along the eastern part of Brazil from Rio Grande do Sul to Paraíba (Moure and Kerr, 1950). Traditionally, two subspecies have been recognized, *M. quadrifasciata anthidioides* and *M. quadrifasciata quadrifasciata*, based upon yellow stripes on the second to fifth tergites, which are continuous in *M. q. quadrifasciata* and interrupted in *M. q. anthidioides* (Ducke, 1916; Schwarz, 1932).

M. q. quadrifasciata is found in southern São Paulo, Paraná, Santa Catarina, and Rio Grande do Sul (Kerr, 1951; Moure, 1975), whereas *M. q. anthidioides* ranges from northern and northeastern São Paulo, along eastern

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Brazil, to Paraíba (Kerr, 1951). Kerr (1951) and Moure (1975) show a hybridization zone between the two subspecies in the state of São Paulo and southern Minas Gerais, where intermediary patterns of yellow tergal stripes are detected. Recently, Batalha-Filho et al. (2009) described the range distribution of the two populations with continuous stripes and inhabiting clearly disjunct areas in relation to the main distribution of the subspecies *M. q. quadrifasciata*: one in northern Minas Gerais and another in northeastern Bahia and Sergipe.

According to Batalha-Filho et al. (2009), the geographic distribution of *M. quadrifasciata* is associated with regions higher than 500 meters above sea level, except for its southern range limit where lower elevation is counterbalanced by higher latitude. Such relationship can be exemplified by the distribution of this species in the state of Espírito Santo, where *M. quadrifasciata* is found only in the upper mountain portion of the state, being unreported on northern sites, such as the Atlantic rainforest in Bahia. The populations of *M. quadrifasciata* bearing continuous tergal stripes on northeastern Bahia and Sergipe are associated with the São Francisco river mouth in low elevation regions.

Some molecular studies have been carried out in *M. quadrifasciata*. Waldschmidt et al. (2000) analyzed the subspecies of *M. quadrifasciata* and detected a RAPD-PCR marker exclusive to *M. q. quadrifasciata* and absent in *M. q. anthidioides*. Waldschmidt et al. (2002), also using RAPD markers in populations of *M. quadrifasciata*, observed two genetically distinct groups, one being composed of *M. q. quadrifasciata* and other by *M. q. anthidioides*. Different mtDNA restriction patterns were detected between both subspecies by Moreto and Arias (2005). Souza et al. (2008) analyzed 155 colonies of both subspecies of *M. quadrifasciata* using RFLP in the cytochrome b gene and found an association between the RFLP pattern and the difference in tergal stripes of each subspecies. Populations exhibiting continuous abdominal stripes from northern Minas Gerais, Sergipe, and northeastern Bahia, however, were not included in that study. Two colonies from northern Minas Gerais, exhibiting continuous tergal stripes, were analyzed by Waldschmidt et al. (2000, 2002) and they were genetically similar to *M. q. anthidioides*. However, in those studies the relationship between the pattern of tergal stripes and genetic similarity was not addressed and the sampling was limited.

Here we analyze a large number of sampling sites which cover, for the first time, most of the species range, including localities sampled for the first time. Furthermore, the populations with continuous tergal stripes recently described by Batalha-Filho et al. (2009) were also included.

Some molecular population genetics studies have been conducted with bees (Tanaka et al., 2001; Soucy and Danforth, 2002; Dick et al., 2004; Cruz et al., 2006; Quezada-Euán et al., 2007; Kraus et al., 2008). However, none of the studies have applied coalescent and more recent phylogeographic methods.

The goal of the present study was to elucidate the phylogeographic pattern and demographic history of *M. quadrifasciata*. We address two main questions: (i) is there correlation between the distribution of tergal stripes pattern and the maternal mitochondrial lineage? In other words, do populations with continuous tergal stripes form a monophyletic group? (ii) What demographic event(s) in Neotropical region explain the observed phylogeographic pattern? The present results will contribute to our knowledge about the biogeographic and evolutionary patterns of this taxon and the Neotropical region.

2. MATERIAL AND METHODS

2.1. Sampling and DNA extraction

Samples (N = 145 colonies, one worker per colony) were collected between 2002 and 2008 in 56 localities in the Brazilian states of Bahia (BA), Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), Rio Grande do Sul (RS), Santa Catarina (SC), and Sergipe (SE) (Tab. S1 available in online material and Fig. 1). The samples were stored in absolute ethanol at –80 °C until DNA extraction.

Samples were divided into four groups based on the pattern of tergal stripes: (i) subspecies *M.*

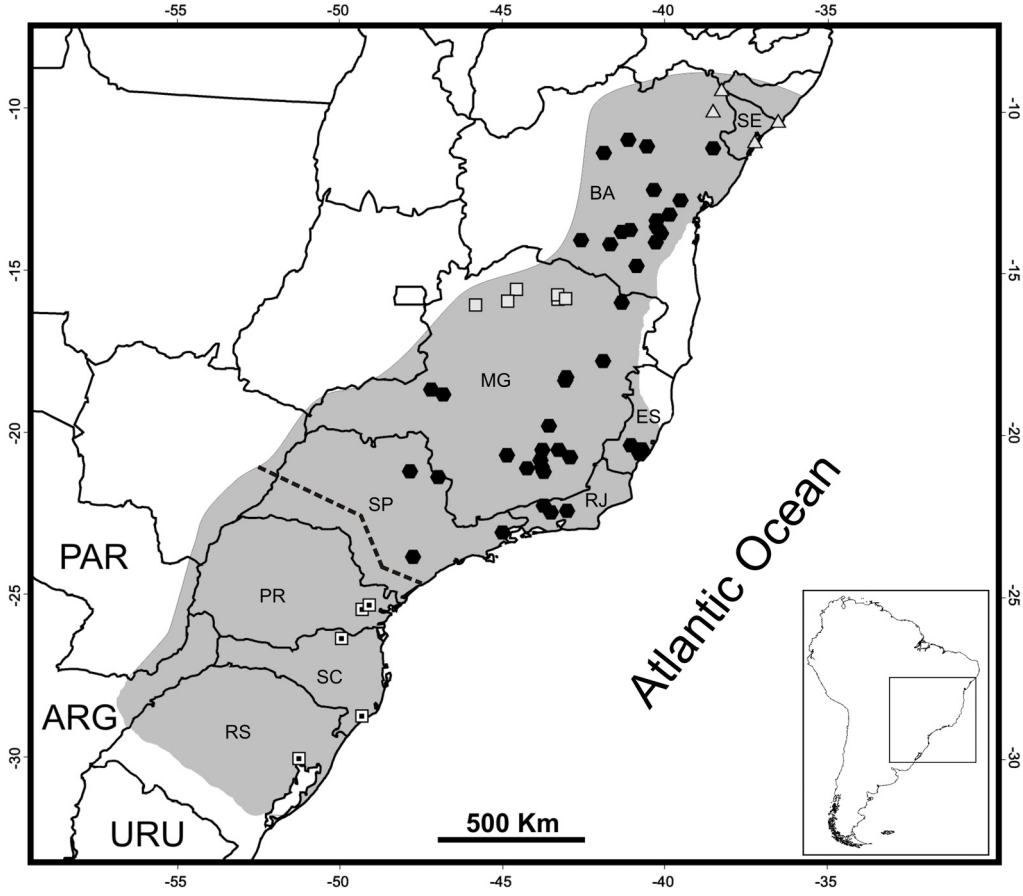

Figure 1. Map of the geographic distribution of the 56 sampling sites of *M. quadrifasciata*. The white squares with black points indicate individuals of *M. q. quadrifasciata*, the black hexagons indicate individuals of *M. q. anthidioides*, the white squares indicate the individuals with continuous tergal stripes from northern Minas Gerais, and the white triangles indicate the specimens with continuous tergal stripes from Sergipe and northeastern Bahia. The grey area represents the geographical range of *M. quadrifasciata* as determined by Batalha-Filho et al. (2009). The dashed line represents the putative barrier to gene flow estimated with the software SAMOVA 1.0.

q. quadrifasciata exhibiting continuous stripes; (ii) subspecies *M. q. anthidioides* with interrupted tergal stripes, intermediate form between interrupted and hybrid, and hybrid form; (iii) *M. quadrifasciata* with pattern of continuous tergal stripes from northern Minas Gerais; (iv) *M. quadrifasciata* with pattern of continuous tergal stripes from Sergipe and northeastern Bahia (Fig. 1).

As outgroups, we selected the species *M. orbignyi* (N = 1) from Campo Grande in Mato Grosso do Sul and *M. subnitida* (N = 1) from Natal in Rio Grande do Norte, which, just like *M. quadrifasciata*, also belong to the subgenus *Melipona* (*Melipona*). The species *M. mandacaia* (N = 2), one sample from Lapão and another from Uibaí (both in the state of Bahia), was included in the phylogenetic reconstruction in order to test the close relationship with *M. quadrifasciata*, as reported by Silveira et al. (2002) and Camargo and Pedro (2007).

Total DNA was extracted from head and thorax of *M. quadrifasciata* specimens as described by Fernandes-Salomão et al. (2005). The samples were electrophoretically separated in 0.8% agarose gel to verify the amount and the quality of extracted DNA. All DNA samples analyzed in this study was

deposited in the DNA bank at the Molecular Biology Laboratory at Universidade Federal de Viçosa. Genbank as accession numbers are provided in Table S1 available in online material.

2.2. Amplification and sequencing

We amplified 1051 bp of the cytochrome oxidase subunit 1 gene (COI) from mitochondrial DNA using primers COX2 (5′ CAATTACTATATTATTATTTGATCG 3′) and COX4 (5′ CTTGAAATGAAATTATATTTCATGTTG 3′) (present study). The PCR reaction (25 μL) was composed of 1 μL of template DNA (50 ng), 5 μL of 5x buffer (Promega), 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of each primer at 20 μM, 2 μL of dNTPs at 100 mM and 1 U of *GoTaq*® DNA polymerase (Promega). Amplifications were performed as follows: an initial denaturation step at 94 °C for 5 minutes; followed by 35 cycles at 94 °C for 1 minute, 52 °C for 1 minute and 20 seconds and 64 °C for 2 minutes; plus a final extension step at 64 °C for 10 minutes. Amplified DNA was purified with the enzyme ExoSap-IT® (USB Corporation) and directly sequenced in both directions (forward and reverse), in an automated sequencer MegaBace DNA Analysis System 500 (Amersham Biosciences) using the kit DYEnamic ET Dye Terminator Cycle Sequencing (Amersham Biosciences).

2.3. Sequence alignment and phylogeographic inferences

The chromatograms were edited using Phred, Phrap, Consed package (Ewing et al., 1998; Ewing and Green 1998; Gordon et al., 1998). Sequences were aligned using the CLUSTAL W method (Higgins et al., 1994) available in the software MEGA4 (Tamura et al., 2007) and compared to the complete COI sequence of *M. bicolor* (Silvestre et al., 2008). All alignments were inspected and corrected visually.

Haplotype (Hd) and nucleotide (π) diversities were estimated with the software DnaSP 4.0 (Rozas et al., 2003). The substitution model adopted in the Bayesian phylogenetic reconstruction was estimated using MrMODELTEST (Nylander et al., 2004), while the substitution model used in the maximum-likelihood approach was calculated using MODELTEST (Posada and Krandall, 1998). The Akaike information criterion (AIC), including outgroups, was used to estimate the substitution models.

Phylogenetic relationships were estimated by Bayesian inference, neighbor-joining, and maximum likelihood. The Bayesian phylogenetic inference analysis were done using MrBAYES 3.1 (Huelsenbech and Ronquist, 2001) with 3 000 000 Markov chain Monte Carlo (MCMC) generations. The first 750 000 generations were excluded as burn in, and the posterior probability were estimated from the remaining trees. The neighbor-joining tree was calculated using the software MEGA4 (Tamura et al., 2007) based on the substitution model of Tamura-Nei with 1000 bootstrap replications. The maximum-likelihood approach was calculated using the NNI heuristic search model with 1000 bootstrap replications, in the software PAUP* (Swofford, 1998). The Bayesian and maximum-likelihood topologies were visualized using the software TREEVIEW 1.6.6 (Page, 2001).

A haplotype network was obtained for analysis of phylogeographic structure using the median-joining network method (Bandelt et al., 1999) in the software NETWORK 4.5.0.0 (www.fluxus-engineering.com).

Analysis of molecular variance (AMOVA, Excoffier et al., 1992) and the estimation of a possible barrier to gene flow were carried out using the method of spatial molecular variance analysis (SAMOVA) in the software SAMOVA 1.0 (Dupanloup et al., 2002). AMOVA was calculated with three hierarchic levels and two groups. Thirty-eight populations were discriminated by this analysis as shown in Figure 3.

In order to verify the demographic expansion and reveal population bottlenecks, the mismatch distribution and the neutrality tests Tajima's D (Tajima, 1983), Fu's F_s (Fu, 1997), and R_2 (Ramos-Onsins and Rozas, 2002) were performed on the obtained phylogroups using the software DnaSP 4.0 (Rozas et al., 2003). Neutrality tests significance was determined based on 1000 coalescent simulations.

Coalescence among clades was estimated using the software MDIV (Nielsen and Wakeley, 2001). This program relies on Markov-chain Monte Carlo simulations to estimate the maximum-likelihood of three parameters; θ ($2N_e\mu$), M (N_em), and T (t/N_e), where N_e is the effective population size, μ is the substitution rate, m is the migration rate, and t is the divergence time among populations. Four simultaneous analyses with different random seeds were performed, each one with 5 000 000 cycles and

Table I. Summary statistics for southern and northern clades and in the species as a whole.

	Hd/sd	π/*sd*	*D*	F_s	R_2
Southern clade	0.879 ± 0.046	0.00268 ± 0.00046	–1.27175	–3.568**	0.0409**
Northern clade	0.944 ± 0.008	0.00334 ± 0.00018	–1.88573*	–35.259*	0.0334*
Total	0.957 ± 0.006	0.00547 ± 0.00037	–1.60475	–35.614*	0.0410**

Hd, haplotype diversity; π, nucleotide diversity; *sd*, standard deviation; *D*, Tajima test; F_s, Fu test; R_2, Ramos-Onsins and Rozas test; $^{**}P < 0.01$ and $^{*}P < 0.05$.

a burn-in of 1 250 000, assuming a $M_{MAX} = 10$ and a $T_{MAX} = 5$ and using the HKY substitution model. Parallel simulations using different M_{MAX}, T_{MAX} values and chain cycles yielded convergent values, showing that the number was appropriate. To calculate the divergence time, a substitution rate calibrated for other insects (Caccone and Sbordoni, 2001; Farrel, 2001) and used in Euglossini bees (Dick et al., 2004) was adopted. Assuming that COI varies uniformly, the minimum rate for 852 bp equals $\mu = 1.02 \times 10^{-5}$ and the maximum rate is $\mu = 1.28 \times 10^{-5}$ substitutions per year. We assumed a generation time of 1 year (Nogueira-Neto, 1954). We calculated confidence intervals around the parameter estimates using Akaike information criterion (AIC) (Burnham and Anderson, 2002) to determine the range of the divergence time from values that were not significantly less likely than the best estimated value (Nielsen and Wakeley, 2001). We accepted values within 2 AIC units on either side of the most likely estimated parameter value.

3. RESULTS

3.1. Characteristics of the mtDNA sequences

Sequences of 852 bp of the COI mitochondrial gene were obtained from 145 specimens of *M. quadrifasciata*, 2 specimens of *M. mandacaia* (with the same haplotype, FJ975765), 1 specimen of *M. orbignyi* (FJ975767), and 1 specimen of *M. subnitida* (FJ975768). According to the alignment in *M. quadrifasciata*, 55 sites were variable, corresponding to 6.45%; 32 of these sites were parsimoniously informative and 23 were singletons. Eight out of the 55 variable sites referred to the first base within the codon, 4 referred to the second base, and 43 to the third base. The variable sites were responsible for 10 amino acid substitutions. The nucleotide composition was: T = 42.5%, C = 9.4%, A = 37.3% and G = 10.7%. Fifty haplotypes were identified (Tab. S1 available in online material), with a nucleotide diversity (π) of 0.00547 and haplotype diversity (Hd) of 0.957 (Tab. I).

3.2. Evolutionary relationships in *M. quadrifasciata*

The evolutionary model selected for the Bayesian inference, including the outgroups, was GTR + I based on the AIC with a proportion of invariable sites of 0.8286. For the maximum-likelihood, including outgroups, the selected model was TIM + I based on the AIC with a proportion of invariable sites of 0.8283. The topologies obtained by the three methods of phylogenetic reconstruction were similar. Figure 2 shows the topology based on the Bayesian inference. Two clades with high values of bootstrap supporting the branches were identified; one regarding the northern distribution and another related to the southern range of *M. quadrifasciata* (Fig. 2). The southern clade comprises the subspecies *M. q. quadrifasciata* and ranges from the state of Paraná to Porto Alegre in the state of Rio Grande do Sul, presenting 10 haplotypes. The northern clade is composed of the subspecies *M. q. anthidioides* and two populations with continuous tergal stripes, one from northern Minas Gerais another from Sergipe and northeastern Bahia, ranging from São Paulo up to the northern limit of geographical distribution, comprising 40 haplotypes.

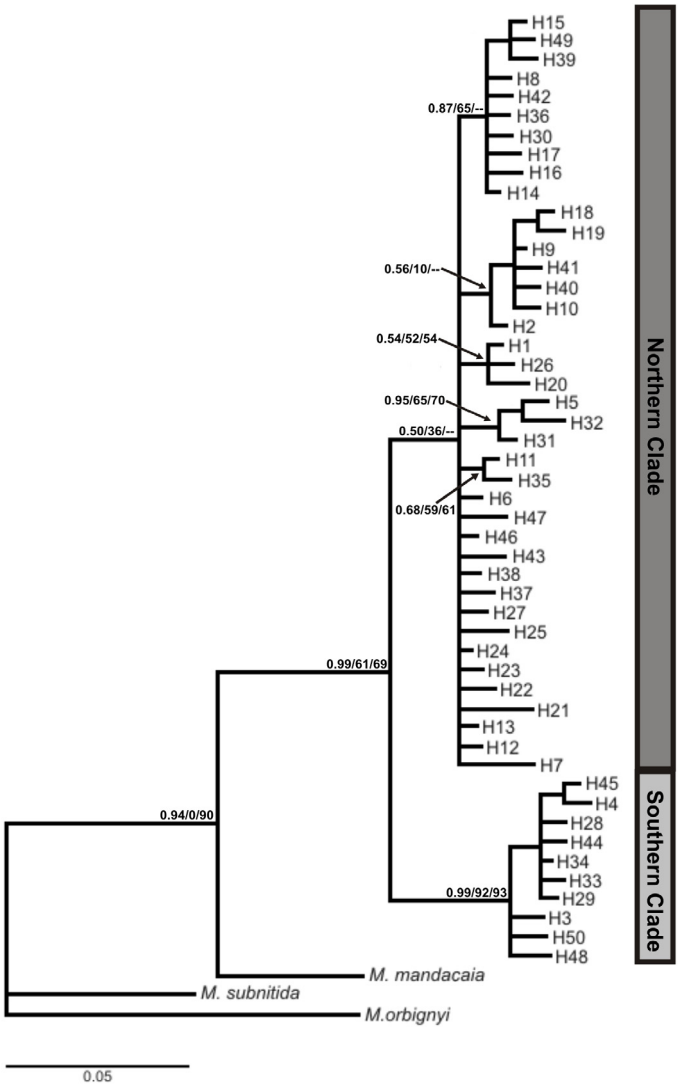

Figure 2. Bayesian inference using the GTR + I model for the COI gene in *M. quadrifasciata* and outgroups. The haplotypes codes are according to those from Table S1 available in online material. The branch support is expressed as a posteriori Bayesian probabilities and bootstrap values for neighbor-joining and maximum likelihood, respectively.

3.3. Geographic structure

The haplotype network agrees with the phylogenetic reconstruction, indicating the presence of two clades (southern and northern), separated from each other by 6 mutational steps (Fig. 3). In the southern clade, corresponding to *M. q. quadrifasciata*, no geographical structure was detected for the analyzed haplotypes. The haplotypes H29 and H34, the most frequent ones within this clade, are found in distinct localities such as Curitiba (H34), Itaiópolis (H34), Içara (H29), and Porto Alegre (H29 and H34). On the other hand,

some degree of geographical structure seems to occur within the north clade, involving a few mutational steps.

The central haplotype in the network (H24), found in the northern clade, is present in Viçosa, Oliveira, and Caeté, all located in the state of Minas Gerais (MG). The small geographic structure was observed in some localities (Fig. 3). A geographical structure was observed in the south portion of Espinhaço Hills in Minas Gerais related to nearby localities, with the formation of one group bearing the haplotypes H1, H20, and H26, identified in Barbacena, Cristiano Otoni, and Ressaquinha, and another presenting the haplotypes H5, H31, and H32, comprising the localities of Piranga and Diogo de Vasconcelos. However, São João Del Rey shares a haplotype identified in the state of Rio de Janeiro (H13).

The SAMOVA, comprising three hierarchic levels showed a structure composed of two groups, in agreement with the phylogenetic reconstruction and the haplotype network, with a percentage variation between southern and northern clades of 68.56% (Tab. II).

3.4. Structure in the pattern of tergal stripes

No evidence of genetic structure in relation to the pattern of tergal stripes was found. Although a structure comprising six mutational steps was detected for *M. q. quadrifasciata*, the populations with continuous stripes from northern Minas Gerais, Sergipe, and northeastern Bahia were unstructured and remained clustered with *M. q. anthidioides* in the northern clade (Fig. 3).

Nevertheless, exclusive haplotypes were observed in the populations with continuous tergal stripes from Sergipe and northeastern Bahia, even though the most frequent haplotype (H38) is separated by a single mutational step from the central haplotype present in *M. q. anthidioides* within the north clade (H24) (Fig. 3).

The populations bearing continuous stripes from northern Minas Gerais shared the haplotype H14 with *M. q. anthidioides* from Bahia. The localities Ourolândia and Nova Soure in the state of Bahia, although displaying a hybrid pattern of tergal stripes also presented the haplotype H14 (Tab. S1 available in online material and Fig. 3).

3.5. Demographic history

Barrier to gene flow as estimated through SAMOVA was located near the Ribeira do Iguape Valley, southwards in the state of São Paulo and agrees with the separation between north and south clades presently studied (Fig. 1).

The unimodal distribution in the pairwise differences observed by mismatch distribution for each clade showed a geographic expansion in both north and south clades (Fig. S1A and S1B, respectively, available in online material), suggesting the occurrence of a population bottleneck in both groups. Neutrality tests suggested that both clades of *M. quadrifasciata* expanded recently (Tab. I).

3.6. Coalescence analysis and time of divergence

Coalescence estimative using the software MDIV generated the probabilities of the parameters θ, M and T (Fig. S2 available in online material). The values with the highest probabilities are $\theta = 12.46$, $M = 0.02$ and $T = 0.81$ (Tab. S2 available in online material). The low M value (0.02) indicates remote gene flow between clades. The divergence time (t) estimated, assuming a minimum $\mu = 1.02 \times 10^{-5}$ and a maximum $\mu = 1.28 \times 10^{-5}$ rates, was 0.493 (0.292–0.840) and 0.394 (0.233–0.672) million years ago, respectively (Tab. S2 available in online material).

4. DISCUSSION

Phylogenetic analyses and haplotype network divided *M. quadrifasciata* into two clades, a northern and a southern clade. Such discrimination fits the subdivision between both subspecies; *M. q. quadrifasciata* is adapted to cold climates while *M. q. anthidioides* lives in tropical environments (Kerr,

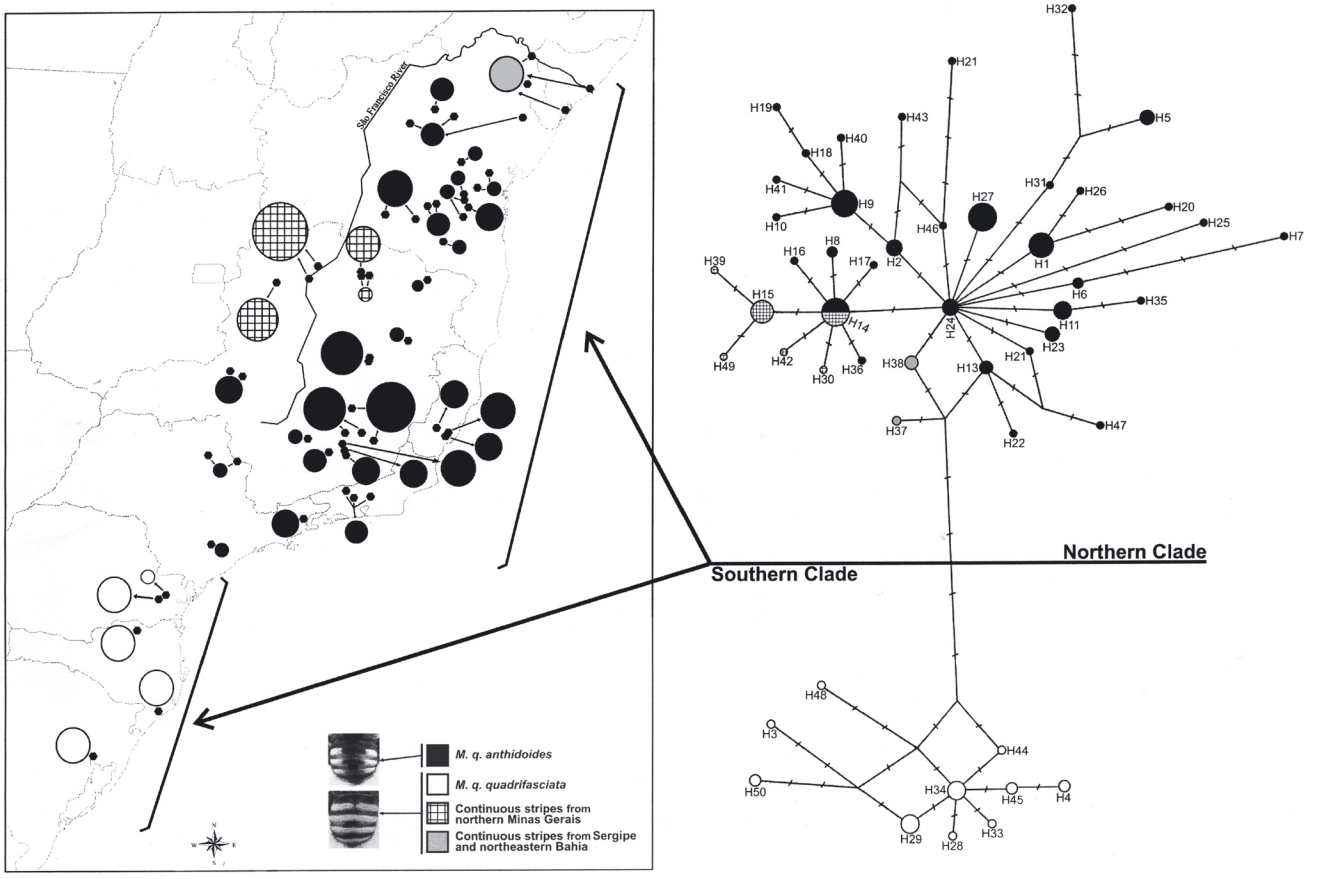

Figure 3. Map of the geographical distribution of populations and median-joining haplotype network of the COI gene in *M. quadrifasciata*. Each circle in the map represents one population as delimited by this study; the size of the circle represents the frequency of individuals and the black hexagons represent the sampled sites. Each circle in the haplotype network corresponds to one haplotype and its size is proportional to the haplotype frequency; each mark in the line connecting the haplotypes refers to a mutational step. The haplotype code is according to tab. S1 available in online material.

Table II. Analysis of molecular variance (AMOVA) in *M. quadrifasciata*. The groups were estimated by SAMOVA.

Variation source	D.F.	Variation %	Φ	*P*-value
Among groups	1	68.56	$\Phi_{CT} = 0.698$	< 0.00001
Among localities within groups	36	21.95	$\Phi_{ST} = 0.905$	< 0.00001
Within localities	106	9.49		
Total	143			

1951; Moure, 1975). However, this adaptation to divergent climatic conditions has probably occurred after the vicariance between both clades.

A high structure between clades was revealed by AMOVA, Φ_{ST} and Φ_{CT} values (Tab. II). The estimated barrier to gene flow is located close to Ribeira do Iguape Valley, southern state of São Paulo, in agreement with the separation between the southern and the northern clade. These results also support the division inferred from the phylogenetic tree and haplotype network.

It is known that inferences using mtDNA estimates of population size, gene flow, population growth, and divergence times are associated with large bias (Edwards and Beerli, 2000). However, Avise (2008) showed that despite the lack of concordance between mtDNA and nucDNA, the matrilineal structure itself would mean that each geographic location is essentially autonomous with regard to reproductive output. Yet, Zink and Barrowclough (2008) defended the utility of mtDNA, highlight its ability to capture patterns of population division because of its lower effective population size, and that the use of mtDNA leads to accurate conclusions regarding taxonomic relationships in the great majority of the time.

The lack of structure within the southern clade might be a consequence of a recent bottleneck event and a reduced population expansion, once the geographical range of this clade is more restricted when compared to that in the northern clade.

The genetic structure observed in the northern clade possibly stems from recent isolation by distance. The structure observed in the southern part of Espinhaço Hills in Minas Gerais is possibly related to recent refuges (final Pleistocene and Holocene) associated with the myriad of mountains found throughout the region. Within the northern clade, the species is distributed over distinct biomes, including high Atlantic rainforest, gallery forests in cerrado (Brazilian savanna), and arborous caatinga (semi-arid region). Such heterogeneity was possible responsible for increasing the diversity within the northern clade. However the reduced sample size of the southern clade in relation to northern clade may have influenced these results.

The low nucleotide diversity (Tab. I), neutrality tests (Tab. I), and the unimodal distribution with few pairwise differences in the mismatch distribution (Fig. S1 available in online material) suggest the occurrence of population bottlenecks followed by recent demographic expansion.

Analysis of the phylogeographic structure related to the pattern of tergal stripes showed no structure regarding such distinctive pattern (Fig. 3). However, a structure in the haplotype network was found in *M. q. quadrifasciata* (south clade) involving six mutational steps in relation to the other populations. The populations exhibiting a continuous pattern of tergal stripes from northern Minas Gerais, Sergipe, and Northeastern Bahia, along with the populations of *M. q. anthidioides*, composed the northern clade.

Souza et al. (2008) analyzed populations of *M. quadrifasciata* by cytochrome b PCR-RFLP found an association between the RFLP profiles and the pattern of tergal stripes in the subspecies of *M. quadrifasciata*. Such association was absent in the present study, once specimens with distinct stripe patterns shared a same haplotype (H14) (Fig. 3).

Presence of continuous tergal stripes in populations from northern Minas Gerais,

Sergipe, and northeastern Bahia is likely to represent an ancestral polymorphism, since the continuous stripe pattern in the subgenus *Melipona* (*Melipona*) seems to be a plesiomorphic trait, shared among all bees of this group distributed throughout Brazil (G.A.R. Melo, unpubl. data).

Specimens with a hybrid pattern of tergal stripes from Ourolândia and Nova Soure in the state of Bahia also had the haplotype H14, which is primarily found in either *M. q. anthidioides* or in populations with continuous tergal stripes from northern Minas Gerais. Such structure indicates that the hybrid stripe pattern resulted from hybridization between *M. q. anthidioides* and the populations with continuous stripes from Sergipe and northeastern Bahia and is not related to maternal inheritance. Nonetheless, the hybridization process might involve gene flow between males with continuous tergal stripes and females with interrupted stripe pattern. Further analyses using nuclear genes might eventually answer this question.

There are bee species occurring in sympatry that presenting interrupted metasomal stripes pattern similar to *M. q. anthidioides*. Yet, this subspecies is widely distributed when compared to the one bearing continuous tergal stripes. According to Silveira and Martines (2009) the presence of interrupted metasomal stripes in some species may be related to a mimicry complex.

Coalescence analysis indicated that the north-south vicariance probably took place between 0.233 and 0.840 million years ago. This dating agrees with the more recent movement estimated for the Guapiara lineament, during the Quaternary (>1.3 million years ago) (Melo et al., 1989, Saadi et al., 2002).

The Ribeira do Iguape Valley where the barrier to gene flow was estimated presents a Quaternary geological fault known as Guapiara lineament (Saadi et al., 2002). According to Melo et al. (1989) and Saadi et al. (2002), this lineament is a regular fault whose tectonic activity has remained throughout all the Quaternary, characterized by deformations in the terraces along Ribeira do Iguape Valley. Geological faults likely determined deep alterations in the forest biota, giving rise to geographical barriers that triggered off the vicariant events over several species (Pereira and Almeida, 1996).

The vicariance between northern and southern observed in the present study may be directly related to the association of *M. quadrifasciata* with higher regions. Geographical distribution data show that this bee is usually absent in regions where the elevation is lower than 500 m (Batalha-Filho et al., 2009). The quaternary fault, represented by the Guapiara lineament in the Ribeira do Iguape Valley, coupled with the phylogeographic rupture herein detected, has putatively promoted the formation of a deep valley (low elevation) that would act as a geographic barrier, giving rise to the formation of northern and southern clades in the studied species.

Relationship between geographical range and elevation seems to be important for some bee species. Silveira et al. (2002) observed disjunction patterns associated with elevation in some species from southern and southeastern Brazil. These observations suggest that such species have been widely distributed in the past during cold periods and, when the temperature increased, they became extinct in lower areas and surviving as population isolates in climatic refuges on the upper parts (Silveira et al., 2002).

Similar phylogeographic patterns have been found in endemic vertebrates of the Atlantic rainforest, like snakes (Grazziotin et al., 2006), birds (Cabanne et al., 2007, 2008), and amphibians (Carnaval et al., 2009). Cabanne et al. (2007, 2008) and Carnaval et al. (2009) reported recent vicariance and demographic expansions associated with forest fragmentation due to climatic changes and agree with the refuge hypothesis during the Pleistocene and paleoclimatic modelling of predicted habitat stability in the Atlantic Forest estimated by Carnaval and Moritz (2008). Although, *M. quadrifasciata* is not endemic of the Atlantic rainforest (also occurring in "Caatinga" biome), the vicariance observed also may be related to the Pleistocene refuge hypothesis.

Small geographical barriers might be effective as a barrier to gene flow for meliponins. According to Nogueira-Neto (1954), the setting of a new nest comprises short distances

since the daughter colony depends on workers and food provided by the original colony up to its complete establishment.

According to Tuomisto (2007), distinct taxa might present similar phylogenetic trees if the speciation is related to vicariance, because the resident species would have undergone a collapse in the ancestor distribution in a similar way, when obstacles to dispersal were formed. However, a similar biogeographical pattern may also arise if different parts of the area have different environmental conditions, which themselves originated in a historical sequence of geological and climatic events (Tuomisto, 2007).

The present study shows that the morphological trait regarding the pattern of stripes on tergites is not a good indicator of differentiation between the subspecies of *M. quadrifasciata*. Notwithstanding, we used mitochondrial data and the tergal pattern may reflect nuclear variation. However, the present data does not reject the subspecies status, since two clades were detected in agreement with previous observations carried out by Kerr (1951) and Moure (1975).

Studies about the evolutionary diversification in the Neotropical region are extremely important to help us understand the role of Pleistocene glaciations and geomorphological alterations in the biota observed at present. Further analyses are necessary to evaluate whether the vicariance effects herein detected have affected other bee species or other animal groups similarly.

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Phylogéographie et démographie historique de l'abeille sans aiguillon néotropicale *Melipona quadrifasciata* (Hymenoptera, Apidae) : manque de correspondance entre morphologie et ADN mitochondrial.

biogéographie / coalescence/ rayures / tergite / *Melipona quadrifasciata* / sous-espèce / ADN mitochondrial/ Brésil

Zusammenfassung – Phylogeographie und historische Demographie der neotropischen stachellosen Biene *Melipona quadrifasciata* (Hymenoptera, Apidae): Gegensätze zwischen Morphologie und mitochondrialer DNA. Stachellose Bienen gehören zu den häufigsten und ökologisch bedeutsamsten wirbellosen Tieren in tropischen Lebensgemeinschaften. Die neotropische stachellose Biene *Melipona quadrifasciata* hat zwei Unterarten: *M. quadrifasciata quadrifasciata* und *M. quadrifasciata anthidioides*. Das hauptsächliche Unterscheidungsmerkmal dieser beiden Unterarten sind gelbe metasomale Streifen, die bei *M. q. quadrifasciata* durchgehend, und bei *M. q. anthidioides* unterbrochen sind. Die Art ist entlang der Ostküste Brasiliens verbreitet, wobei *M. q. quadrifasciata* im Süden und *M. q. anthidioides* im Norden des Verbreitungsgebiets vorkommt.Vor kurzem wurden zwei Populationen mit zusammenhängenden Streifen beschrieben, die nördlich des von *M. q. quadrifasciata* besiedelten Gebietes in disjunkten Arealen vorkommen.
In dieser Studie untersuchen wir die populationsgenetische Struktur und die historische Demographie von *M. quadrifasciata* mit Hilfe von Sequenzen der mitochondrialen DNA aus umfangreichem Probenmaterial (Abb. 1 und Tab. S1, online material). Dabei untersuchen wir zwei Hauptfragen: (i) korreliert die morphologische Variation (Verteilung des Streifenmusters auf dem Abdomen) mit der Variation der mitochondrialen DNA? Mit anderen Worten, stammen die Populationen mit zusammenhängenden Streifen aus derselben mütterlichen Linie ab? (ii) Welche demographischen Ereignisse können die beobachteten phylogeographischen Muster erklären?
Zur Beantwortung dieser Fragen sequenzierten wir 852 bp der Untereinheit1 des Cytochromoxidase (CoI) Gens aus der mitochondrialen DNA von 145 Völken (1 Arbeiterin pro Volk), die entlang des Verbreitungsgebiets der Art gesammelt worden waren (Abb. 1). Die Ergebnisse von phylogeographischen Tests und Koaleszenzberechnungen machten deutlich, dass es zwei unterschiedliche Gruppen gibt: eine südliche Klade, die aus *M. q. quadrifasciata* besteht, und eine nördliche, die *M. q. anthidioides* sowie die beiden disjunkten Populationen mit durchgehenden Streifen beinhaltet. Interessanterweise sind also Formen, die durchgehende Streifen gemeinsam haben, nicht

unbedingt monophyletisch; umgekehrt haben Individuen mit verschiedener Morphologie denselben mtDNA Haplotypen gemeinsam (H14 in Abb. 3). Wir schlagen vor, die durchgehenden tergalen Streifen in den diskjunkten Populationen als einen anzestralen Polymorphismus zu betrachten, da nach den Ergebnissen anderer Studien das Muster aus zusammenhängenden Streifen innerhalb der Untergattung *Melipona* (*Melipona*) ein plesiomorphes Merkmal zu sein scheint. Der Zeitpunkt der Divergenz der beiden Gruppen wurde auf die Zeit des Pleistozäns, zwischen 0,233 und 0,840 Millionen Jahren vor der Gegenwart bestimmt (online material, Abb. S2 und Tab. S1), die durch Klimaänderungen und geomorphologische Umbildungen in der neotropischen Region charakterisiert war. Ähnliche phylogeographische Muster wurden bei endemischen Wirbeltieren des Atlantischen Regenwaldes nachgewiesen, zum Beispiel bei Schlangen, Vögeln und Amphibien.

Biogeographie / Koaleszenz / Tergale Streifen / *Melipona quadrifasciata* / Unterart

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